

We claim:

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1. A method for selectively labeling or tagging phosphate groups in a natural or synthetic peptide or protein in the presence of carboxylic acid groups which comprises the steps of:

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a. reacting the natural or synthetic peptide or protein with a protective group that reacts to protect the phosphate groups therein by forming phosphoramido bonds and to protect the carboxylic acid groups therein by forming amide bonds;

b. treating the protected peptide or protein under conditions which selectively substantially cleave the phosphoramido bond, without substantially cleaving the amide bond to regenerate free phosphate groups in the peptide or protein; and

c. reacting the free phosphate groups in the peptide or protein, in which the carboxylic acids groups remain protected, with a label or tag comprising a functional group that reacts with a phosphate.

2. -The method of claim 1 wherein the protecting group of step a is an amine.

3. The method of claim 1 wherein the label or tag is a solid phase material and a free phosphate group of the oligomer or polymer is covalently linked to the solid phase material directly or indirectly through a linker moiety.

4. The method of claim 1 wherein the amino group is reacted with the phosphate group using a carbodiimide catalyzed reaction.

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Sub C1 is this an additional step?

5. The method of claim 1 wherein the free phosphate groups are reacted with a linker group having a phosphate reactive group and a second reactive group that functions for forming a covalent bond to the solid phase material. *of c²*

6. The method of claim 5 wherein the second reactive group is a sulfhydryl reactive group.

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7. The method of claim 1 wherein the amine protective group is ethanolamine.

8. The method of claim 7 wherein in step b the protected peptide or protein is treated with trifluoroacetic acid to selectively regenerate free phosphate groups.

9. The method of claim 1 wherein the free phosphate groups are reacted with a linker containing a sulfhydryl group or a latent reactive group that can be transformed into a sulfhydryl group. *no "tag" added?*

Links what?

10. The method of claim 9 wherein the free phosphate group is reacted with cystamine and a free sulfhydryl group is generated by reduction of cystamine. *a tag?*

11. The method of claim 10 wherein the reducing agent is DTT.

15 12. The method of claim 9 wherein in step c the peptide or protein is covalently attached to a solid support material through reaction with the sulfhydryl group of the linker. *linker must be attached to*

13. The method of claim 12 wherein the solid support material is glass beads with immobilized iodoacetyl groups.

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DECODE

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support, p 30, etc

14. The method of claim 1 wherein the natural or synthetic peptides or proteins are obtained from a tryptic digest.
15. The method of claim 1 wherein the label or tag is a radiolabel, a fluorescent label, a colorimetric label or an affinity label.
16. The method of claim 1 wherein the label or tag is an affinity label.
17. The method of claim 1 wherein the label is a reactive label. *dupl. of 1*
18. The method of claim 1 further comprising a step of detecting the selectively labeled phosphopeptide.
19. The method of claim 18 wherein the phosphopeptide is detected by detection of the label.
20. The method of claim 18 wherein the label or tag is an affinity label and the phosphopeptide is detected by binding to a corresponding capture reagent.
21. The method of claim 1 further comprising a step of separating selectively labeled phosphopeptides from the mixture by attachment of the phosphopeptides to a solid support or by binding of the phosphopeptides to a capture reagent.
22. A method for detecting one or more phosphopeptides in one or more samples containing a mixture of peptides which comprises the steps of:

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- a. selectively protecting the carboxylic acid groups of the peptides in the one or more samples such that any phosphate groups in the peptides remain unprotected;
- b. selectively labeling the unprotected phosphate groups in the peptides in the sample with a label having a functional group that reacts directly or indirectly with a phosphate; and
- c. detecting the peptides carrying the label to detect the phosphopeptides in the sample.

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23. The method of claim 22 wherein the carboxylic acid groups of the peptides are selectively protected by initial reaction with a protecting group that protects both carboxylic acid groups and phosphate groups in the peptides followed by selective deprotection of the phosphate groups in the peptides.
(invn't C 22 dupl. of 17)

24. The method of claim 23 wherein the carboxylic acid groups and phosphate groups of the peptides are initially protected with an amine group that forms amide bonds with carboxylic acid groups and phosphoramido bonds with phosphates and wherein the phosphate groups are selectively deprotected by selective cleavage of the phosphoamido bonds.

25. The method of claim 24 wherein the amide bonds and phosphoramido bonds are formed by a carbodiimide-catalyzed condensation reactions.

26. The method of claim 25 wherein the phosphate groups are selectively deprotected without cleavage of the amide bonds by treatment with mild acid.

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27. The method of claim 22 wherein the label is a radiolabel, a fluorescent label, a colorimetric label or an affinity label.

28. The method of claim 22 wherein the label is an affinity label and the phosphopeptide is detected by binding to a corresponding capture reagent.

5 29. The method of claim 28 wherein the label is a reactive label.

30. The method of claim 29 wherein the reactive label carries a reactive group that can form a covalent bond to a solid phase material.

31. The method of claim 29 wherein the reactive label carries a latent reactive group.

32. The method of claim 22 further comprising the step of separating selectively labeled peptides prior to detection step c.

15 33. The method of claim 22 wherein the label carries a reactive group that can form a covalent bond to a solid phase material and wherein the selectively labeled peptides are separated by first covalently attaching the labeled peptides to the solid phase material, then washing the solid support to remove peptides that are not covalently attached to the support and thereafter releasing peptides from the solid phase material.

34. The method of claim 33 wherein the peptides released from the solid phase material are detected using mass spectrometric techniques.

35. The method of claim 34 wherein tandem mass spectrometry is used to detect the peptides.

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36. The method of claim 35 wherein tandem mass spectrometry is further used to determine the amino acid sequence of peptides and the precise position of the phosphorylated amino acid within the peptide sequence.

37. The method of claim 1 for detecting one or more phosphopeptides in two or more samples wherein differentially isotopically labeled carboxylic acid protecting groups are employed with different samples.

38. The method of claim 37 for detecting one or more phosphopeptides in two or more samples wherein the labels employed in different samples are differentially isotopically labeled.

39. The method of claim 37 wherein tandem mass spectrometry is used to detect the one or more phosphopeptides and the relative amounts of phosphopeptides in the two or more samples is determined by measuring the relative amounts of the differentially isotopically labeled labels present.

40. The method of claim 39 wherein combined microcapillary liquid chromatography and tandem mass spectrometry are employed to detect the peptides.

41. The method of claim 1 wherein the amine group is a hydroxy amine.

42. ~~The method of claim 41 wherein the amine group is ethanolamine.~~

43. The method of claim 1 wherein the protecting group comprises a combination of differentially isotopically labeled protecting groups.
Support

20 44. ~~The method of claim 43 wherein the protecting group is a hydroxy acid.~~

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45. The method of claim 43 wherein the protecting groups are differentially isotopically labeled using deuterium.

46. The method of claim 22 for detecting one or more phosphopeptides in two or more samples wherein differentially isotopically labeled ethanolamine is used to protect the carboxylic acid groups of peptides in different samples.

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47. The method of claim 22 further comprising a step of determining the sequence of one or more phosphopeptides detected.

48. The method of claim 47 wherein the sequence of the phosphopeptide is determined by tandem mass spectrometry.

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49. The method of any of claims 48 wherein the samples are protein digests containing peptides and the sequence of the phosphopeptide detected is used to identify the protein from which the phosphopeptide is derived.

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50. The method of claim 22 in which the amount of one or more proteins in a sample is also determined by mass spectrometry, and which further comprises the step of introducing into a sample a known amount of one or more internal standards for each of the proteins to be quantitated.

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51. The method of claim 50 in which different samples represent proteins expressed in response to different environmental or nutritional conditions, different chemical or physical stimuli or at different times.

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52. The method of claim 22 wherein different samples are labeled with different fluorescent labels and the relative intensity of a labeled peptide in different samples

(1) can be measured by measuring the relative intensity of the fluorescence emission of the different labeled peptides.

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53. A kit for selectively labeling phosphopeptides in a mixture of peptides which comprises:

5 (a) a protective group which reacts with a carboxylic acid or ester thereof and which also reacts with a phosphate group; and

 (b) a mild acid reagent for selectively regenerating any free phosphate groups in the peptide by reacting the protected peptides under sufficiently mild acid conditions such that the phosphoramido bond is substantially cleaved and the amide bond is substantially not cleaved.

54. The kit of claim 53 wherein the protective group is an amine.

55. The kit of claim 53 wherein the protective group comprises differentially isotopically labeled protective groups.

15 56. The kit of claim 53 further comprising a radiolabel, a fluorescent label, a colorimetric label or an affinity label.

57. The kit of claim 56 wherein the label is an affinity label and the phosphopeptide is detected by binding to a corresponding capture reagent.

58. The kit of claim 56 wherein the label is a reactive label.

Sub
B2

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Dose
Limitation

Sub
B3

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Dose
Limitation

Sub
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B4

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Dose
Limitation

Sub
A34
B4

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Dose
Limitation

59. The kit of claim 56 wherein the reactive label carries a reactive group that can form a covalent bond to a solid phase material.
60. The kit of claim 56 wherein the reactive label carries a latent reactive group.
61. The kit of claim 53 further comprising iodoacetylated glass beads.
62. The kit of claim 53 wherein the label carries a reactive group that can form a covalent bond to a solid phase material and wherein the selectively labeled peptides are separated by first covalently attaching the labeled peptides to the solid phase material, then washing the solid support to remove peptides that are not covalently attached to the support and thereafter releasing peptides from the solid phase material.
63. The kit of claim 53 further comprising one or more solid support materials.
64. The kit of claim 53 further comprising a reagent for removing covalently linked phosphopeptides from a solid support.
65. The kit of claim 53 further comprising one or more enzymes for carrying out a peptide digest.
66. The kit of claim 53 which comprises:
 - one or more enzymes for carrying out a peptide digest;
 - one or more protective groups that react with carboxylic acid or carboxylic acid ester groups and with a phosphate group;
 - one or more reagents for selectively cleaving a phosphoramido bond in the presence of amide bonds to generate free phosphates; and

one or more labels which carry a functional group for selectively reacting with free phosphate groups.

67. The kit of claim 53 which comprises:

one or more enzymes for carrying out a peptide digest;

one or more protective groups that react with carboxylic acid or carboxylic acid ester groups and with a phosphate group;

one or more reagents for selectively cleaving a phosphoramido bond in the presence of amide bonds to generate free phosphates; and

one or more linkers which carry a functional group for selectively reacting with free phosphate groups and carry a reactive group or a latent reactive group for reacting with a solid support material;

one or more solid support materials to which the reactive group or the latent reactive group can be attached; and

one or more reagents for cleaving a phosphopeptide from a solid support material.